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14. ABSTRACT

Mutations in TSC1 and TSC2 genes are responsible for the majority of tuberous sclerosis complex (TSC). The major function of TSC1/2 is to inhibit mTORC1. Therefore, uncontrolled mTORC1 activation is a key molecular basis for TSC and mTORC1 inhibitors is being used for TSC related complication. We have focused our efforts on the molecular mechanisms of negative regulation of mTORC1 by upstream signals. We have shown that mTORC1 is potently inhibited by hormones that stimulating cAMP. We found that cAMP acts through protein kinase A (PKA) to inhibit mTORC1. Our data indicate that PKA phosphorylates Raptor, a subunit if mTORC1, to inhibit mTORC1. We observed the mTORC1 is potently inhibited by osmotic stress and a possible role of the NLK kinase in mTORC1 inhibition. We also discovered that different amino acids stimulate mTORC1 by different mechanisms.

15. SUBJECT TERMS
TSC, mTOR, cAMP, PKA.

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Micro(RNA) Managing by mTORC1

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In this issue of *Molecular Cell*, Ye et al. (2015) demonstrate that mTORC1 globally regulates miRNA biogenesis under nutrient-rich conditions via the E3 ubiquitin ligase Mdm2, which promotes Drosha degradation.

Mechanistic target of rapamycin (mTOR) is a conserved protein kinase and a component of the mTOR complex 1 (mTORC1). mTORC1 senses multiple stimuli, such as nutrients and growth factors, to control a variety of downstream pathways involved in metabolism and cell growth (Zoncu et al., 2011). Cells and organisms grow when conditions are favorable and nutrients are plentiful. mTORC1 coordinates nutrient availability with cell growth by stimulating anabolic processes like protein synthesis and by inhibiting cellular catabolism through autophagy repression in nutrient rich conditions (Jewell et al., 2013). Recently, chronic treatment of cancer cells with the potent mTORC1 inhibitor rapamycin was shown to alter microRNA (miRNA) profiles (Sun et al., 2010; Totary-Jain et al., 2013). However, the mechanistic link between mTORC1 and miRNA biogenesis was unknown. In this issue, Ye et al. (2015) fill in the missing gap by providing evidence that nutrients, such as glucose and amino acids, regulate global miRNAs through mTORC1. Specifically, nutrient-induced mTORC1 activation increases the levels of the E3 ubiquitin ligase Mdm2, which ubiquitinates and targets the miRNA-processing enzyme Drosha for proteasomal-dependent degradation (Figure 1). Degradation of Drosha results in reduced miRNA processing and global downregulation of steady-state miRNA levels. These new findings emphasize the impact that nutrients and the cellular environment have on miRNA biogenesis and compliment results observed in mouse studies, where maternal diet was shown to alter a subset of miRNAs in the offspring through mTORC1 (Alejandro et al., 2014).

The human genome encodes some 1000 miRNAs, and dysregulation of miRNAs is often associated with many human diseases, particularly cancer (Mendell and Olson, 2012). miRNAs are a class of small non-coding regulatory RNAs that are ~21–22 nucleotides in length and function in RNA silencing and post-transcriptional regulation of gene expression. The generation of miRNAs is achieved by two RNase III-type endonucleases Drosha and Dicer. miRNA biosynthesis is under tight spatial control that starts in the nucleus with the synthesis of a long transcript known as primary miRNA (pri-mRNA). Drosha and its interacting partner DiGeorge syndrome critical region gene 8 (DGCR8) process the pri-miRNA to a precursor miRNA (pre-miRNA), and the pre-miRNA is then exported from the nucleus into the cytoplasm by exportin-5. Dicer-dependent processing converts the pre-miRNA to mature miRNA, which unites with the Argonaute (Ago) family of proteins within the RNA-induced silencing complex (RISC). RISC utilizes the miRNAs as guide to silence post-transcriptional genes (Ha and Kim, 2014). Understanding how the cellular environment, such as nutrients, controls the basic machinery involved in miRNA biogenesis is of great interest in biology research.

Considering the importance of both mTORC1 and miRNAs in cancer development, it is perhaps not surprising that some crosstalk between them exists. The results by Ye et al. (2015) reveal the intricate molecular details involved in this crosstalk by uncovering an mTORC1-Mdm2-Drosha pathway that regulates global miRNA biogenesis. Nutrient-induced mTORC1 activation appears to increase Mdm2 mRNA and protein levels. However, the precise mechanism by which mTORC1 controls Mdm2 levels is not clear. The increase in Mdm2 mRNA suggests that mTORC1 regulates Mdm2 at the transcriptional level. Therefore, it seems likely that mTORC1-dependent phosphorylation of a transcriptional regulator of Mdm2 may be involved. Furthermore, Mdm2 has not been reported to be a substrate for mTORC1. Is Mdm2 phosphorylated by mTORC1? Does mTORC1 shuttle into the nucleus to modulate Mdm2 levels? Does mTORC1 regulate Mdm2 protein levels in the cytoplasm, or maybe at the lysosome, where mTORC1 is activated? Interestingly, Mdm2 was identified as a binding partner and an E3 ubiquitin ligase for Drosha. Mdm2-dependent ubiquitination of Drosha targeted Drosha to the proteasome for subsequent degradation. The tumor suppressor p53 is a well-established transcriptional regulator of Mdm2 and has been implicated downstream of mTORC1 regulation (Lee et al., 2007). Thus, the authors investigated if p53 was involved in this signaling cascade. Elevated mTORC1 activity increased Mdm2 mRNA ~10-fold, which was abolished in the absence of p53. However, despite unchanged Mdm2 mRNA levels with high mTORC1 activity in p53 null cells, Mdm2 protein levels were still significantly high when compared with p53 null cells where mTORC1 activity was low. Taken together, the authors conclude that nutrient-induced mTORC1 activation regulates Mdm2 by a p53-dependent transcriptional route and an alternative p53-independent post-transcriptional route. Two distinct pathways downstream of mTORC1 may control Mdm2 levels and global miRNA biogenesis.

In further exploring glucose deprivation through mTORC1, Drosha appeared to be critical for cell sensitivity to apoptosis. Because Drosha levels were significantly elevated under glucose starvation, the authors speculated that it may upregulate miRNAs crucial for cell survival under

Ragulator-binding domain activates mTORC1 signaling even in the absence of amino acids. The activation of mTORC1 by amino acids, particularly arginine, is defective in cells lacking SLC38A9. Given these results and that arginine is highly enriched in lysosomes from at least one mammalian tissue (*29*), we propose that SLC38A9.1 is a strong candidate for being a lysosome-based arginine sensor for the mTORC1 pathway. To substantiate this possibility, it will be necessary to determine the actual concentrations of arginine and other amino acids in the lysosomal lumen and cytosol and compare them to the affinity of SLC38A9.1 for amino acids. If high arginine levels are a general feature of mammalian lysosomes, it could explain why SLC38A9.1 appears to have a relatively broad amino acid specificity; perhaps no other amino acid besides arginine is in the lysosomal lumen at levels that approach its K_m.

The notion that proteins with sequence similarity to transporters function as both transporters and receptors (transceptors) is not unprecedented (*31*, *32*). The transmembrane region of SLC38A9.1 might undergo a conformational change upon amino acid binding that is then transmitted to Ragulator through its N-terminal domain. What this domain does is unknown, but it could regulate Ragulator nucleotide exchange activity or access to the Rag GTPases by other components of the pathway. To support a role as a sensor, it will be necessary to show that amino acid binding regulates the biochemical function of SLC38A9.1.

Even if SLC38A9.1 is an amino acid sensor, additional sensors, even for arginine, are almost certain to exist, as we already know that amino acid–sensitive events exist upstream of Folliculin (*15*, *33*) and GATOR1 (*34*), which, like Ragulator, also regulate the Rag GTPases. An attractive model is that distinct amino acid inputs to mTORC1 converge at the level of the Rag GTPases, with some initiating at the lysosome through proteins like SLC38A9.1 and others from cytosolic sensors that remain to be defined (Fig. 5G). Indeed, such a model would explain why the loss of SLC38A9.1 specifically affects arginine sensing but its overexpression makes mTORC1 signaling resistant to arginine or leucine starvation: Hyperactivation of the Rag GTPases through the deregulation of a single upstream regulator is likely sufficient to overcome the lack of other positive inputs. A similar situation may occur upon loss of GATOR1, which, like SLC38A9.1 overexpression, causes mTORC1 signaling to be resistant to total amino acid starvation (*14*).

Modulators of mTORC1 have clinical utility in disease states associated with or caused by mTORC1 deregulation. The allosteric mTOR inhibitor rapamycin is used in cancer treatment (*35*) and transplantation medicine (*36*). However, to date, there have been few reports on small molecules that activate mTORC1 by engaging known components of the pathway. The identification of SLC38A9.1—a protein that is a positive regulator of the mTORC1 pathway and has an amino acid binding site—provides an opportunity to develop small-molecule agonists of mTORC1 signaling. Such molecules should promote mTORC1-mediated protein synthesis and could have utility in combatting muscle atrophy secondary to disuse or injury. Lastly, a selective mTORC1 pathway inhibitor may have better clinical benefits than rapamycin, which in long-term use inhibits both mTORC1 and mTORC2 (*37*). SLC38A9.1 may be an appropriate target to achieve this.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S6
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METABOLISM

Differential regulation of mTORC1 by leucine and glutamine

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The mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) integrates environmental and intracellular signals to regulate cell growth. Amino acids stimulate mTORC1 activation at the lysosome in a manner thought to be dependent on the Rag small guanosine triphosphatases (GTPases), the Ragulator complex, and the vacuolar H^+–adenosine triphosphatase (v-ATPase). We report that leucine and glutamine stimulate mTORC1 by Rag GTPase-dependent and -independent mechanisms, respectively. Glutamine promoted mTORC1 translocation to the lysosome in RagA and RagB knockout cells and required the v-ATPase but not the Ragulator. Furthermore, we identified the adenosine diphosphate ribosylation factor–1 GTPase to be required for mTORC1 activation and lysosomal localization by glutamine. Our results uncover a signaling cascade to mTORC1 activation independent of the Rag GTPases and suggest that mTORC1 is differentially regulated by specific amino acids.

Cells sense environmental nutrient flux and respond by tightly controlling anabolic and catabolic processes to best coordinate cell growth with nutritional status. The mechanistic target of rapamycin (mTOR), a conserved serine-threonine kinase, is part of the mTOR complex 1 (mTORC1), which helps coordinate cell growth with nutritional status. Dysregulation of mTORC1 is common in human diseases, including cancer and diabetes (*1*). Amino acids are essential for mTORC1 activation (*2*, *3*); however, it remains unclear how specific amino acids are sensed. Leucine (Leu) (*2*, *4*, *5*), glutamine (Gln) (*5*–*7*), and arginine (Arg) (*2*) have been implicated in

mTORC1 activation. In one model, mTORC1 indirectly senses amino acids within the lysosomal lumen that requires the Rag guanosine triphosphatases (GTPases), which are regulated by the pentameric Ragulator complex, the vacuolar H^+–adenosine triphosphatase (v-ATPase), and the Gator complex (*8*, *9*). When activated, the Rag GTPases bind to and recruit mTORC1 to the lysosome, where the Rheb GTPase activates mTORC1 (*4*). In mammals, there are four Rag proteins: RagA and RagB, which are functionally redundant; and RagC and RagD, which are also functionally equivalent. The formation of a heterodimer between RagA or RagB with RagC or RagD, and the guanine nucleotide state of the Rag proteins determines mTORC1 recruitment to the lysosome and subsequent activation (*4*, *10*, *11*). Under amino acid sufficiency, RagA and RagB complexes are guanosine triphosphate (GTP)–loaded and capable of binding Raptor. Somehow the v-ATPase detects the build-up of lysosomal amino acids (*12*), stimulates Ragulator guanine nucleotide exchange factor (GEF) activity, and inhibits Gator GTPase-activating protein (GAP) activity (*9*, *13*). This loads RagA-RagB complexes with GTP and recruits mTORC1 to the lysosome, where it encounters Rheb, a potent mTORC1 activator that mediates growth factor signals. The tuberous sclerosis complex (TSC) tumor suppressor is also localized at the lysosome, and it negatively regulates mTORC1 by acting as a GAP for Rheb (*14*).

We generated mouse embryonic fibroblasts that lack both RagA and RagB [RagA/B knockout (KO) MEFs] (Fig. 1A and fig. S1). RagA-RagB complexes bind directly to mTORC1 (*15*), and overexpression of a constitutively active version of one of the two proteins renders mTORC1 insensitive to amino acid starvation (fig. S2) (*4*, *10*). Deletion of RagA/B diminished the abundance of RagC, consistent with RagA and RagB stabilizing RagC and RagD by forming heterodimers (Fig. 1A) (*4*, *16*). Unexpectedly, deletion of RagA and RagB reduced (~30%), but did not abolish, mTORC1 activity, as judged by the phosphorylation state of its substrates ribosomal S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4E–binding protein 1 (4EBP1). Phosphorylation of S6K1 and 4EBP1 was abolished when the RagA/B KO cells were treated with the mTOR inhibitors Torin1 and Rapamycin or were depleted of the mTORC1 subunit Raptor with short hairpin RNA (shRNA) (fig. S3). Thus, mTORC1 is active in the absence of RagA and RagB.

To investigate the amino acid response of the RagA/B KO MEFs, we stimulated cells with amino acids and analyzed the kinetics of mTORC1 activation. Both the magnitude and rate at which mTORC1 was activated by amino acids were reduced in cells lacking RagA and RagB (Fig. 1B and fig. S4). Likewise, mTORC1 activity was reduced in RagA/B KO MEFs upon amino acid withdrawal (fig. S5). To exclude the possibility that some cells lacking RagA and RagB spontaneously mutated to compensate for decreased mTORC1 activity, we analyzed individual clones derived from the RagA/B KO MEF population. Single clones displayed an increase in mTORC1 activity in response to amino acids (fig. S6). To determine which amino acids activate mTORC1 in the absence of RagA and RagB, we individually stimulated RagA/B KO MEFs with each of the 20 standard amino acids (fig. S7). Leu and Arg stimulated mTORC1 activation in control, but not RagA/B KO cells (Fig. 1C and figs. S7 and S8). Gln-stimulated activation of mTORC1 in RagA/B KO cells displayed kinetics similar to that of control cells and when RagA/B KO cells were stimulated with the 20 standard amino acids (Fig. 1, B and C, and fig. S4). Stable reexpression

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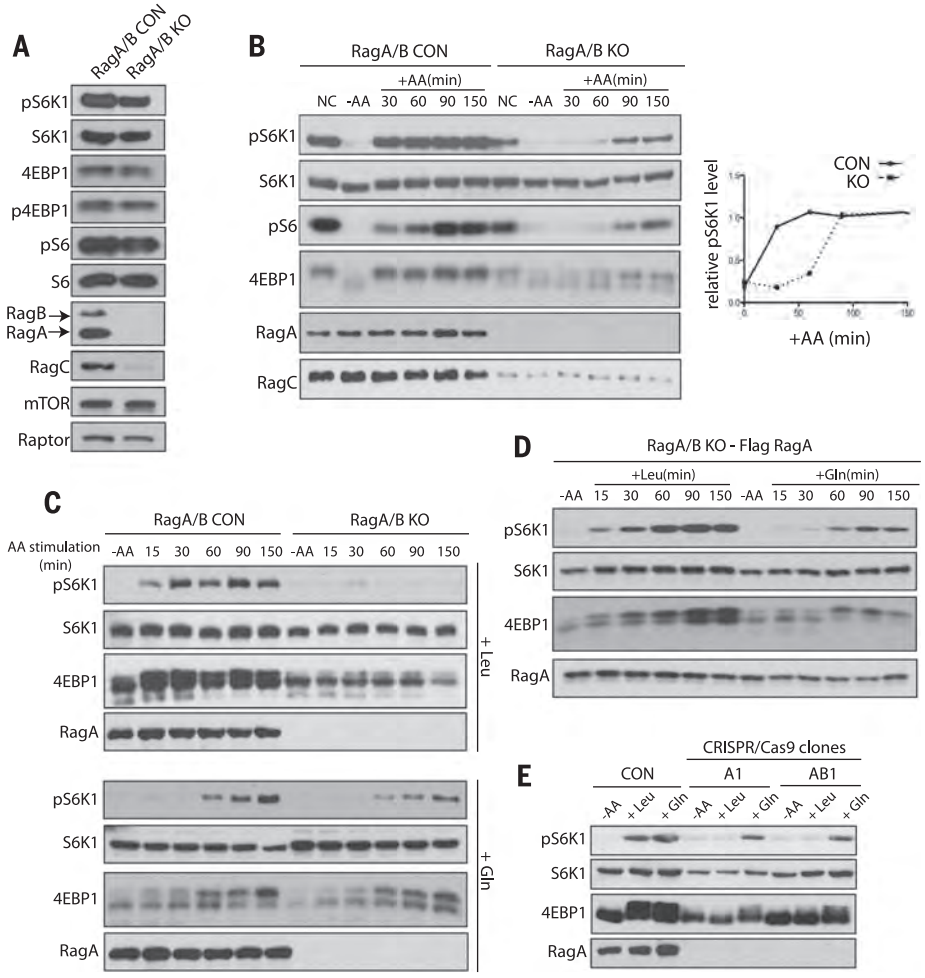

Fig. 1. Gln, but not Leu, activates mTORC1 independently of RagA and RagB. mTORC1 activity was analyzed by the phosphorylation of S6K1 (pS6K1), S6 (pS6), and 4EBP1 (p4EBP1) and the mobility shift of 4EBP1. AA, amino acids. (**A**) mTORC1 activity was analyzed in control (CON) and RagA/B KO MEFs under normal conditions (NC). mTOR, Raptor, RagA, RagB, and RagC protein were also analyzed. (**B**) mTORC1 activity was analyzed in CON and RagA/B KO MEFs under NC, in the absence of amino acids (–AA) and at the indicated times after the addition of amino acids (+AA) (left). Relative abundance of pS6K1 is plotted (right). (**C**) mTORC1 activity after stimulation with Leu (top) or Gln (bottom) in CON and RagA/B KO MEFs. (**D**) mTORC1 activity was analyzed after stimulation with Leu or Gln in RagA/B KO MEFs stably expressing Flag-tagged RagA at the indicated times. (**E**) mTORC1 activity was analyzed in CON, RagA KO (A1) and RagA/B KO (AB1) HEK293A cells that were starved of amino acids or stimulated with Leu or Gln for 150 min.

of Flag-tagged RagA in the RagA/B KO MEFs restored mTORC1 activation in response to Leu (Fig. 1D), which confirmed that RagA and RagB are required for mTORC1 activation by Leu but not Gln.

We performed genome editing by means of clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) to inactivate the RagA and RagB genes in human embryonic kidney 293A (HEK293A) cells (*17*, *18*) (fig. S9). In HEK293A cells and in MEFs, RagA is more abundant than RagB (Fig. 1A and fig. S9E). Loss of RagA alone or both RagA and RagB in these cells prevented Leu-, but not Gln-induced, activation of mTORC1 (Fig. 1E and fig S9F). Thus, Gln can stimulate mTORC1 activation independently of RagA and RagB or cell type.

The lysosome is essential in the amino acid–sensing pathway to mTORC1 and is thought to be a platform for optimal mTORC1 activation that integrates effects of growth factors, such as insulin, through Rheb and those of amino acids through the Rags (*19*). Because Gln can activate mTORC1 in the absence of RagA and RagB (Fig. 1, C and E, fig. S7, and fig. S9F), we investigated whether lysosomal localization of mTORC1 was required for Gln-induced activation of mTORC1 in cells lacking RagA and RagB. In control cells, mTOR translocated to lysosomal membranes identified by the presence of the marker protein lysosome-associated membrane protein 2 (LAMP2) as early as 50 min and remained at the lysosome 150 min after amino acid stimulation (fig. S10A) (*4*, *11*). In contrast, mTOR did not localize to lysosomal membranes in RagA/B KO cells after 50 min of amino acid stimulation (fig. S10B). However, by 150 min, we observed lysosomal localization of mTOR in a subset of cells that also showed activation of mTORC1 (Fig. 2A and fig. S10B). Gln, but not Leu, induced lysosomal localization of mTOR in RagA/B KO MEFs (Fig. 2, B and C). Furthermore, synergistic activation of

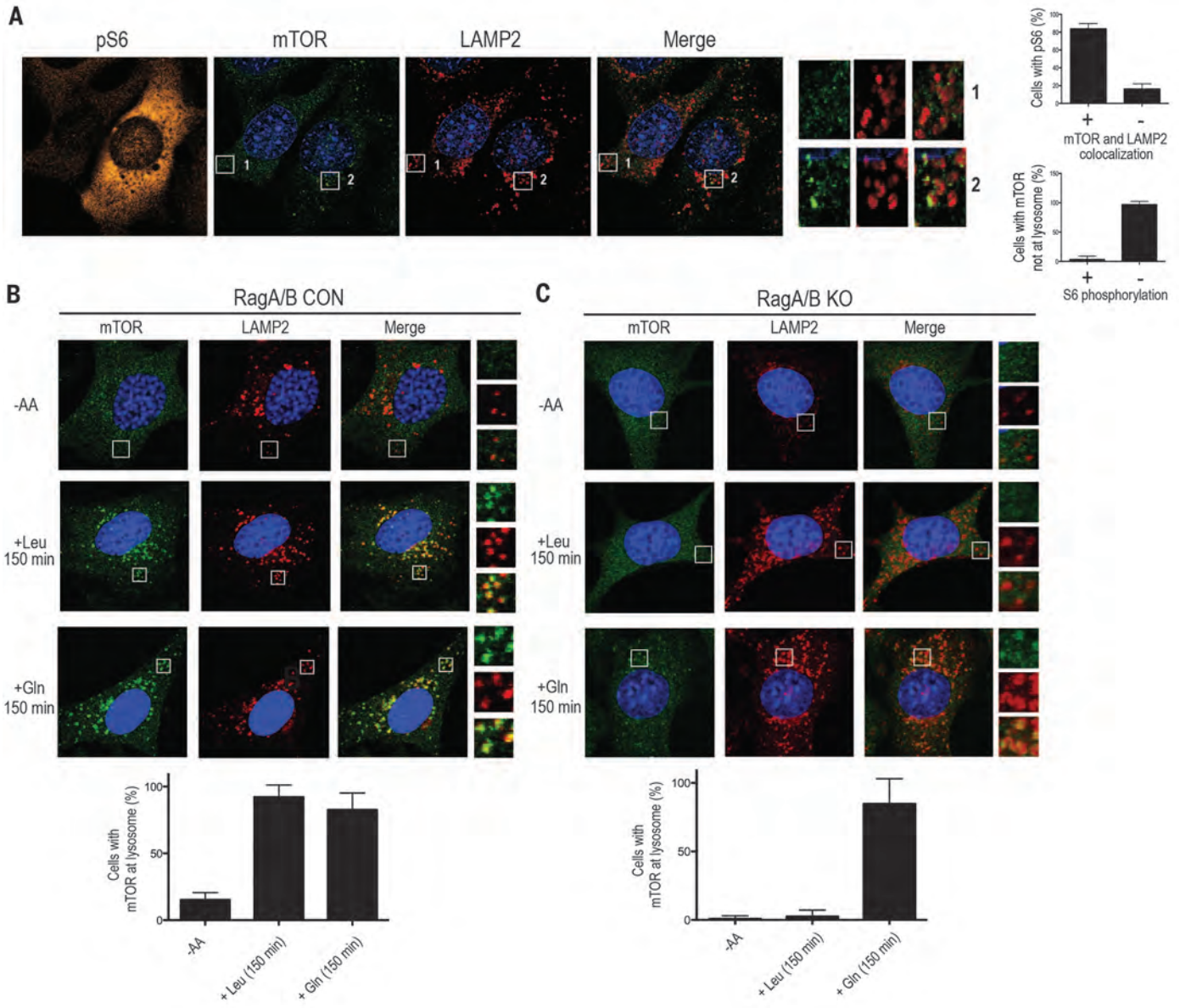

Fig. 2. Gln-induced mTORC1 lysosomal localization in the absence of RagA and RagB. (**A**) Immunofluorescence (IF) analysis depicting mTORC1 activation by phosphorylation of S6 (pS6; orange) in RagA/B KO MEFs. mTOR (green) and LAMP2 (red) are also shown. Quantification of the percentage of pS6 cells with mTOR and LAMP2 colocalization (top right) and the percentage of cells with mTOR not at lysosome that also contain S6 phosphorylation (bottom right). (**B** and **C**) IF analysis depicting mTOR and LAMP2 in CON (B) or RagA/B KO MEFs (C), without amino acids or stimulated with Leu or Gln for 150 min (top). Quantification of the percent of cells with mTOR at the lysosome without amino acids or stimulated with Leu or Gln (bottom). Higher-magnification images (A) to (C) of the area depicted by the inset and their overlays are shown on the right.

mTOR by amino acids and insulin was observed in RagA/B KO cells (fig. S11). Thus, Gln appears to induce mTORC1 activation through translocation to the lysosome in a manner independent of RagA and RagB.

Amino acid transporters (*5*, *12*) and the Ragulator complex (*11*, *13*) have been implicated in mTORC1 activation. We analyzed mTORC1 activity in cells depleted of several amino acid transporters and in MEFs lacking p14 (p14 KO MEFs), an essential subunit of the Ragulator complex. Gln activated mTORC1 in cells depleted of some amino acid transporters and in p14 KO MEFs, which indicated that these transporters and the Ragulator are not required for Gln-induced activation of mTORC1 (fig. S12 and Fig. 3A).

The v-ATPase is essential for the acidification of the lysosome and interacts with the Rags and Ragulator to stimulate mTORC1 activation in response to amino acids (*12*). We treated control and RagA/B KO cells with the v-ATPase inhibitor bafilomycin A (Baf A) (*20*). Baf A inhibited mTORC1 activation in both control and RagA/B KO cells when the cells were stimulated either with all amino acids (Fig. 3B and fig. S13A) or with Leu or Gln individually (Fig. 3C). Baf A also inhibited lysosomal localization of mTORC1 in RagA/B KO cells (fig. S13, B and C). Furthermore, inhibition of the v-ATPase by concanamycin A or inhibition of the lysosomal pH gradient by chloroquine also blocked Gln-induced lysosomal localization and activation of mTORC1 in RagA/B KO cells (fig. S13, D to H). Moreover, depletion of the v-ATPase V0c subunit, which interacts with the Ragulator and controls mTORC1 activity (*12*), largely prevented amino acid–induced activation of mTORC1 in control and RagA/B KO MEFs (Fig. 3D and fig. S13I). Furthermore, depletion of several lysosomal proteins had no effect on Gln-induced activation of mTORC1 and localization in the absence of RagA and RagB, which indicated that modification of the v-ATPase was not secondary to a general disruption in lysosomal structure and function (fig. S13, K and L). Taken together, Gln-induced activation of mTORC1 appears to require the v-ATPase and lysosomal function.

In *Drosophila* S2 cells, TORC1 activity is inhibited in cells depleted of the *Drosophila* ADP ribosylation factor–Arf1 (dArf1) (*21*), and we observed a further decrease in amino acid–induced TORC1 activation when both dRagA and dArf1 were depleted (Fig. 4A). We used small interfering RNA (siRNA) to deplete Arf1 from HEK293A RagA/B KO cells. Gln stimulated mTORC1 activation in RagA/B KO cells treated with a control siRNA; however, it failed to induce mTORC1 activation in RagA/B KO cells depleted of Arf1 (Fig. 4B). Depletion of other Arf family members failed to inhibit Gln-induced activation of mTORC1 in RagA/B KO cells (fig. S14A). Treatment of RagA/B KO cells with brefeldin A (BFA), an Arf1 GEF inhibitor (*22*), at high doses blocked amino acid signaling to mTORC1, whereas BFA caused only a small decrease in mTORC1 activation in response to amino acids in control cells (Fig. 4C and fig. S14, B and C). Consistently, BFA blocked Gln-induced activation of mTORC1 in RagA/B KO cells (Fig. 4D and fig. S14D). In addition, depletion of Arf1 or BFA treatment did not inhibit Leu-induced activation of mTORC1 in control cells, nor did they affect lysosomal pH (fig. S14, E to G).

Leu or Gln stimulation did not appear to affect the guanine nucleotide state of Arf1 (fig. S14H). Overexpression of a constitutively active Arf1-GTP failed to restore mTORC1 activation under amino acid deficiency (fig. S14I). Further, green fluorescent protein–tagged Arf1 (Arf1-GFP) localization was unaffected by amino acid starvation or stimulation (fig. S15). These results indicate that GTP hydrolysis or nucleotide cycling of Arf1, or both, is required for mTORC1 activation.

Arf1 regulates vesicular trafficking, so we tested whether bidirectional inhibition of trafficking between the endoplasmic reticulum (ER) and Golgi would affect Gln-induced activation of mTORC1 (*23*). We depleted proteins involved in anterograde trafficking and treated RagA/B KO cells with Golgicide A (*24*), yet did not observe an effect on Gln-induced activation of mTORC1 (fig. S16). These results support that Arf1 signaling to mTORC1 is specific and independent of ER-Golgi vesicular transport.

RagA/B KO MEFs treated with BFA were analyzed for mTOR localization in response to Gln stimulation. Gln-induced mTOR localization to the lysosome (Fig. 4E and Fig. 2C); however, pretreatment of cells with BFA inhibited the effect of Gln (Fig. 4E). Artificially targeting mTORC1 to the lysosomal surface by adding the C-terminal lysosomal targeting motif of Rheb to Raptor (*11*) activated mTORC1 in RagA/B KO cells, even in the presence of BFA (Fig. 4F). Thus, BFA inhibits mTORC1 by interfering with its lysosomal localization, which implicates Arf1 in the signaling pathway that links Gln to mTORC1 localization and activation at the lysosome.

In conclusion, we show that mTORC1 is differentially regulated by Gln and Leu (fig. S17). Our results demonstrate that RagA and RagB are essential for mTORC1 activation by Leu, but not by Gln, and this appears to be evolutionarily conserved in *Saccharomyces cerevisiae* (*25*). We identified the Arf1 GTPase to be involved in a signaling pathway that connects Gln to mTORC1 activation at the lysosome in the absence of the Rag GTPases. Many cancer cell lines have increased mTORC1 activity and show a high dependence on Gln for growth. Therefore, Gln-induced mTORC1 activation may be important for the growth of both normal and tumor cells.

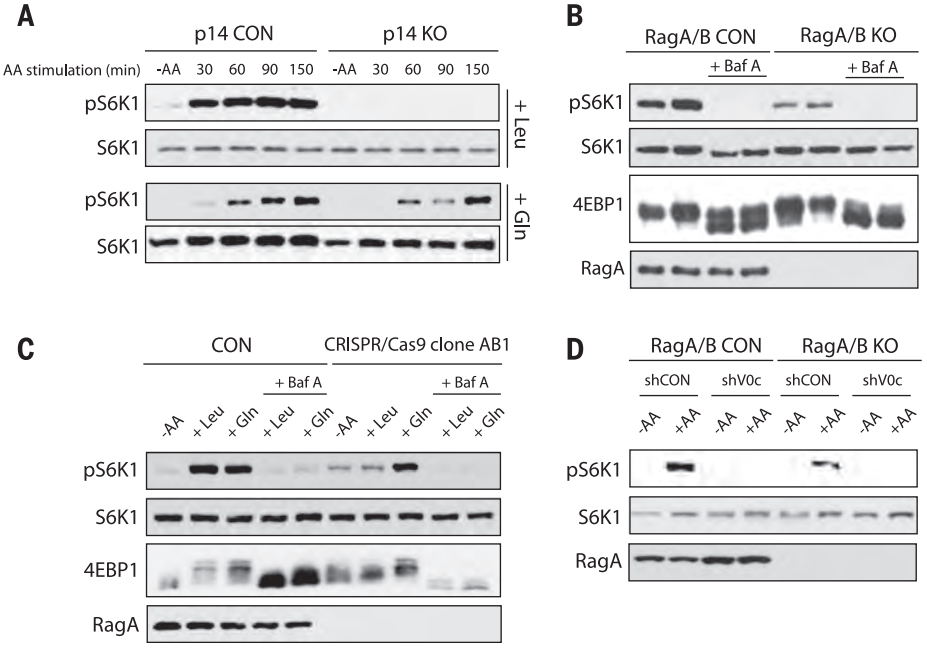

Fig. 3. Gln-induced mTORC1 activation requires the v-ATPase but not the Ragulator. mTORC1 activity was analyzed by the phosphorylation of S6K1 (pS6K) and the mobility shift of 4EBP1. (**A**) mTORC1 activity was analyzed in CON and p14 KO MEFs that were starved of amino acids, then stimulated with Leu (top) or Gln (bottom) at the indicated times. (**B** and **C**) Analysis of mTORC1 activity in CON and RagA/B KO cells that were starved of amino acids; pretreated with or without 1 μM Baf A; followed by amino acid, Leu, or Gln stimulation for 150 min. (**D**) CON and RagA/B KO MEFs were treated with shRNA CON (shCON) or shRNA targeting the v-ATPase V0c subunit (shV0c). CON and RagA/B KO MEFs were starved of amino acids, followed by amino acid stimulation, and mTORC1 activity was assessed.

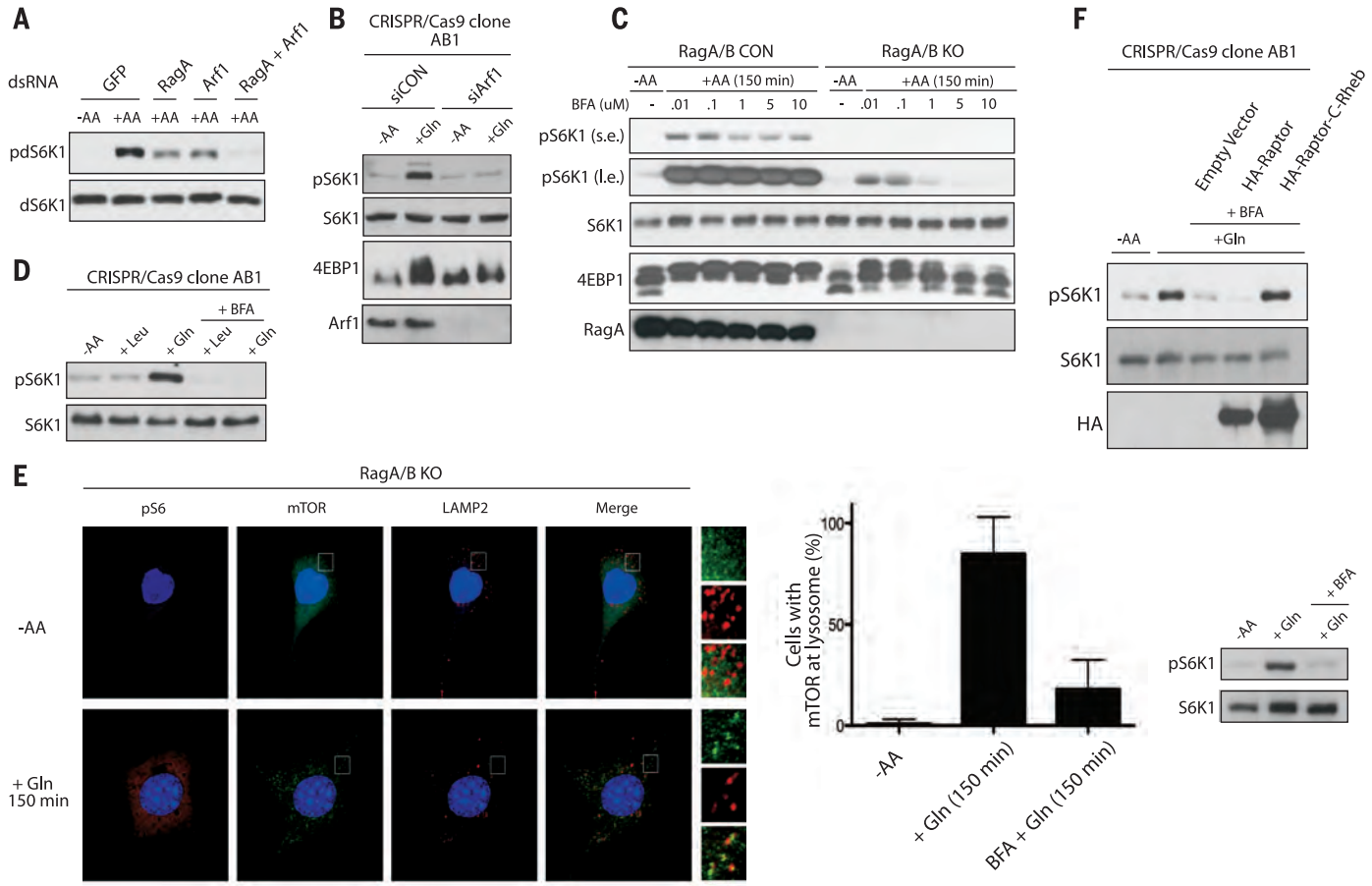

Fig. 4. Arf1 is required for Gln signaling to mTORC1 in the absence of RagA and RagB. (**A**) TORC1 activity was analyzed for the phosphorylation of *Drosophila* S6K1 (pdS6K1) in *Drosophila* S2 cells treated with double-stranded RNA (dsRNA) targeting GFP, RagA, or Arf1. Cells were starved of amino acids for 1 hour, then stimulated with or without amino acids for 30 min. (**B**) mTORC1 activity was analyzed by the phosphorylation of S6K1 (pS6K1) or mobility shift of 4EBP1 in RagA/B KO HEK293A cells treated with control (siCON) or Arf1 siRNA (siArf1). Cells were starved of amino acids then stimulated with Gln for 150 min. (**C**) mTORC1 activity was analyzed as in (B) in CON and RagA/B KO MEFs starved of amino acids, then pretreated with the indicated concentrations of BFA, and stimulated with amino acids for 150 min. Labels s.e. and l.e. denote shorter exposure and longer exposure, respectively. (**D**) mTORC1 activity was analyzed as in (B) in RagA/B KO HEK293A cells starved of amino acids, then pretreated with or without 1 μM BFA, and stimulated with Leu or Gln for 150 min. (**E**) IF analysis depicting mTORC1 activation (pS6; orange) and lysosomal localization (LAMP2; red, mTOR; green) in RagA/B KO MEFs. Cells were starved of amino acids, pretreated with or without 1 μM BFA, followed by stimulation with Gln for 150 min. Higher magnification images of the area depicted by the inset and their overlays are shown on the right of the images. Quantification of the percentage of cells with mTOR at the lysosome under different conditions and corresponding Western blot (right). (**F**) mTORC1 activity was analyzed as in (B) in RagA/B KO HEK293A cells transfected with HA-Raptor or HA-Raptor containing the C-terminal CAAX motif of Rheb (HA-Raptor-C-Rheb). Cells were starved of amino acids, pretreated with or without 1 μM BFA, and stimulated with Gln for 150 min.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S17
References (*26*–*35*)

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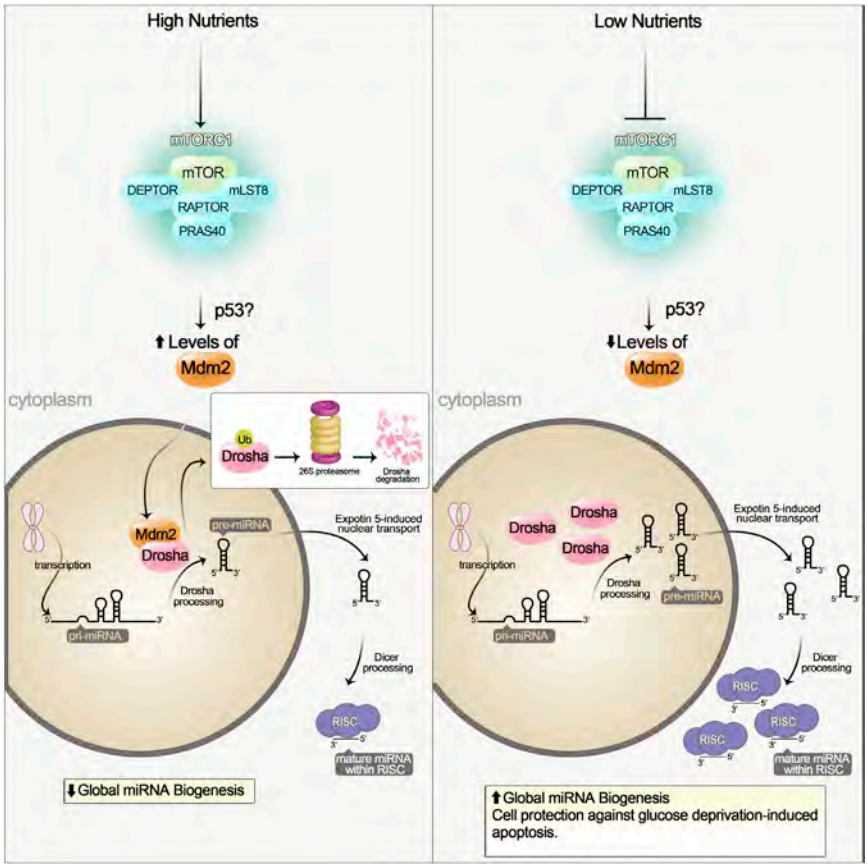

Figure 1. Nutrients Regulate Global miRNA Biogenesis through an mTORC1-Mdm2-Drosha Pathway

(Left) Under nutrient sufficiency mTORC1 is activated, and it increases the levels of the ubiquitin E3 ligase Mdm2. mTORC1 may control Mdm2 levels through a p53-dependent and -independent pathway. Mdm2 ubiquitinates and targets the miRNA-processing enzyme Drosha for proteasomal-dependent degradation. This results in a global decrease of miRNA biogenesis. (Right) Under nutrient deficiency mTORC1 is not active and Mdm2 levels are low. Drosha levels are elevated leading to an increase in global miRNA biogenesis.

such conditions. In fact, silencing Drosha under glucose deprivation increased cell apoptosis, suggesting that miRNA biogenesis may play an essential role in cellular resistance to energy depletion. Performing a high-throughput screen utilizing a miRNA mimic library, which contains double-stranded RNA molecules that mimic native miRNAs, the authors identified four miRNA mimics that could rescue low glucose-induced cell apoptosis when Drosha was silenced. miR-297, miR-376b-3p, miR-567, and miR-627-5p increased resistance of the Drosha-silenced cells to glucose deprivation. Two of the four miRNAs, miR-297 and miR-567, significantly increased Drosha protein levels, suggesting that these two miRNAs may protect cells from apoptosis directly through Drosha levels. Thus, the mTORC1-Mdm2-Drosha pathway appears to play an important role in cellular adaptation to glucose deprivation.

Ye et al. (2015) describe a pathway where nutrients regulate global miRNAs through an mTORC1-Mdm2-Drosha signaling cascade. This study reveals how miRNAs may be regulated or sense environmental signals, such as nutrients. It would be interesting to know if other stimuli, like growth factors or stress, signal through the mTORC1-Mdm2-Drosha pathway to control global miRNA biogenesis. Is mTORC1 activity in general important, or do certain cues that filter through mTORC1 matter? Does Drosha deficiency affect cell survival under serum starvation conditions or in the absence of growth factors? In any event, the results of this study pave the way for new research on the crosstalk between mTORC1 and miRNA biogenesis.

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Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant genetic disorder characterized by the development of benign hamartomas in many organs, such as the brain, kidney, heart, skin, and eyes [1-3]. Mutations in the tumor suppressor genes TSC1 and TSC2 are the major genetic causes for TSC. TSC1 and TSC2 gene products form a physical complex and mainly function to suppress the mechanistic target of rapamycin complex 1 (mTORC1) [4]. TSC1/TSC2 have GTPase-activating protein (GAP) activity to promote GTP hydrolysis and hence inactivation of the small GTPase Rheb [5]. The GTP-bound Rheb can weakly associate with and potently activate mTORC1 on the lysosome. Thus, TSC1/TSC2 suppresses mTORC1 by inactivating the Rheb GTPase. High mTORTC1 activity promotes cellular metabolism, cell growth, and tumorigenesis [4, 6]. Therefore, understanding mTORC1 regulation, both activation and inhibition, is the key to under TSC pathogenesis. Inhibition of mTORC1 has been developed as therapeutic treatment for TSC and related diseases.

Multiple upstream signals act through TSC1/TSC2 to regulate mTORC1 activity and control cell growth [4]. For example, mitogenic growth factors activate AKT to phosphorylate and dissociateTSC2 from the lysosome, where Rheb and mTORC1 are localized [7]. TSC1/2 cannot inhibit Rheb when they are dissociated from the lysosome. On the other hand, amino acids induce mTORC1 translocation to lysosome where it can be activated by the Rheb GTPase [8]. This proposal is based on the observations that elevation of cAMP leads to inhibition of mTORC1. It is worth noting that cAMP displays growth inhibitory effects in most cell types. Therefore, it is possible that cAMP may inhibit mTORC1 to express it cell growth inhibitory function. The major downstream effect of cAMP is the protein kinase A (PKA). A major goal is to understand the mechanism of mTORC1 inactivation by PKA and other cellular stress, such as osmotic stress.

Keywords

Tuberous sclerosis complex, TSC, TSC1, TSC2, mTORC1, amino acid, osmotic stress, oxidative stress, cAMP, GPCR, NLK, Tumor, phosphorylation, kinase, PKA

Overall Project Summary

One major goal of the proposal is to determine how mTORC1 is negatively regulated, particularly in response to elevating cAMP and stress conditions. We showed that cAMP blocks mTORC1 activation by amino acids. We tested the possibility that cAMP may inhibit amino acid transport, thereby to inhibit mTORC1. We have shown that increase of cAMP or manipulation of PKA activity (such as overexpression or PKA inhibitor treatment) did not affect transport of 3H-Leucine, demonstrating that cAMP inhibits mTORC1 by a mechanism independent of amino acid transport. mTORC1 is known to be activated on lysosome [8]. We performed immunofluorescence stain and showed that lysosomal localization of mTORC1 is affected by cAMP. In addition, we observed that PKA directly phosphorylates the raptor subunit in mTORC1. Finally, we found the osmotic stress inhibits mTORC1 and this inhibition maybe mediated by the NLK protein kinase.

cAMP blocks both leucine and glutamine signaling to mTORC1.

Amino acids are the most potent activators of mTORC1 [9]. Amino acids stimulate mTORC1 activity by inducing lysosomal localization. This is accomplished by the ability of amino acids to activate the lysosomal Rag GTPases, thereby stimulating the interaction between mTORC1 and the active Rag GTPases. Among all amino acids, leucine and glutamine are particularly important in mTORC1 activation. We have recently showed that leucine and glutamine activates

mTORC1 by two different mechanisms [10]. Leucine acts through the Rag GTPases to activate mTORC1. In contrast, glutamine acts in a manner independent of Rag GTPases, but requires the Arf1 GTPase to stimulate mTORC1. It is worth noting the leucine and glutamine represent two different classes of amino acids. Leucine is an essential amino acid, so human cells absolutely requires external leucine for protein synthesis and cell growth. In contrast, glutamine is a non-essentially amino acid. Glutamine is unique that it provides both carbon and nitrogen source for the cell. Furthermore, most cancer cells require high concentration for their growth. Moreover, the TSC mutant cells are highly sensitive to glutamine [11]. We tested the effect of cAMP on mTORC1 activation by leucine and glutamine. We found that cAMP effectively blocks the mTORC1 activation by either leucine or glutamine (Fig.1). The above data suggest that PKA likely acts at a step downstream of Rag or Arf1 to suppress mTORC1.

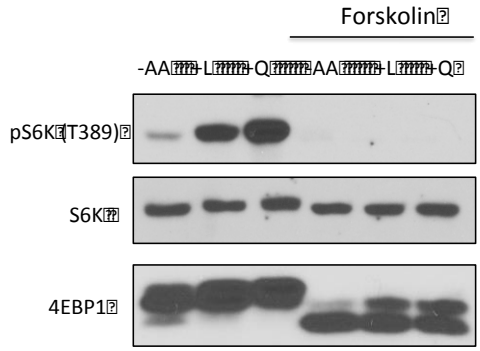

Fig.1. Forskolin inhibits the ability of leucine or glutamine in mTORC1 activation. Cells were cultured in the absence of amino acids (-AA), stimulated with leucine (+L) or glutamine (+Q) for 30minutes as indicated. Treatment with forskolin to increase cAMP is indicated. S6K and 4EBP1 are direct substrates of mTORC1 and their phosphorylation was used as an indirect assay for mTORC1 activity. Western blot for phosphorylation of S6K is detected by the phosphospecific antibody while 4EBP1 phosphorylation can be detected by mobility shift (higher bands are phosphorylated while lower bands are dephosphorylated).

cAMP alters mTORC1 localization

Lysosomal localization is critical for mTORC1 activation because the direct upstream activator Rheb is localized on lysosomal [12]. Amino acids are known to activate mTORC1 by promoting lysosomal localization. We examined the effect of cAMP on mTORC1 subcellular localization. As expected, amino acids induced mTORC1 co-localization with the lysosomal marker LAMP2 (Fig.2, left panels). Interestingly, forskolin and IBMX treatment (which increased cAMP) induced a mTORC1 localization on lysosome regardless the presence or absence of amino acids (Fig.2, right panels). This is a rather surprising result because high cAMP inhibits the mTORC1 activity. It is worth noting that the high cAMP appears to alter the morphology of lysosome. One possibility is that the lysosomal function is altered by cAMP, therefore mTORC1 is inactive even it is on the lysosome. This will be an interesting possibility to be tested in the coming year.

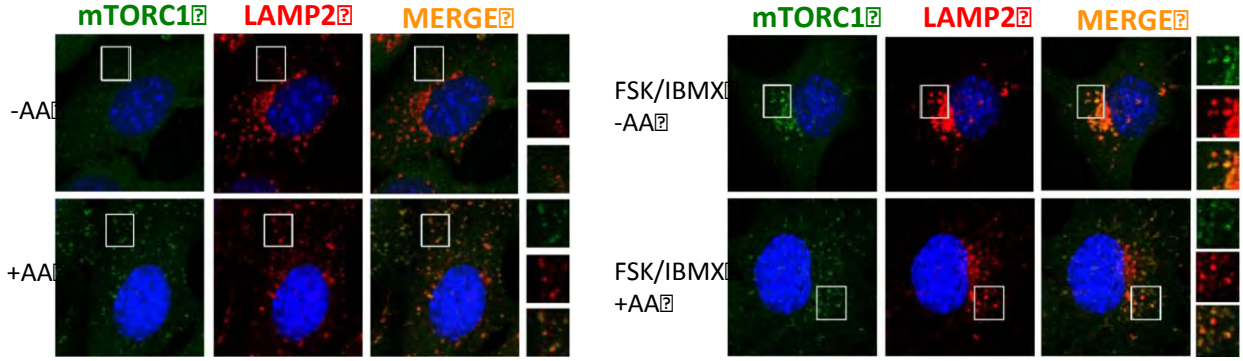

Fig.2. cAMP alters mTORC1 localization. In the control cells, amino acids (+AA) induce co-localization of mTORC1 with the lysosomal marker LAMP2 (left panels). When cellular cAMP is elevated by FSK/IMBX, mTORC1 is co-localized with lysosome even in the absence of amino acid (right panels).

PKA phosphorylates Raptor

We investigated the mechanism of PKA in mTORC1 inhibition. PKA is a protein kinase that normally regulates its downstream targets by phosphorylation. We tested the hypothesis that PKA may inhibit mTORC1 by phosphorylating one of the mTORC1 subunits. The mTORC1 complex was isolated from HEK293 cells by immunoprecipitation with the raptor antibody, which is an essential subunit of mTORC1. The immunopurified mTORC1 was subjected to in vitro phosphorylation by purified PKA in the presence of 32P-ATP and phosphorylation was detected by autoradiography. We found that PKA mainly phosphorylates raptor while other mTORC1 subunits are not phosphorylated (Fig.3). PKA preferentially recognizes substrates with RRXS/T motif (where R for arginine, X for any residue, and S/T for the phosphorylation site serine or threonine) [13]. We search the raptor sequences and find that it contains several putative PKA recognition sites. Mutation of S719 or S791 appears to reduce, but not abolish, raptor phosphorylation by PKA, indicating that they are potential PKA phosphorylation sites in raptor. PKA likely phosphorylation additional sites in raptor. We will investigate the mechanism of raptor phosphorylation by PKA and the functional significance of this phosphorylation in mTORC1 inhibition.

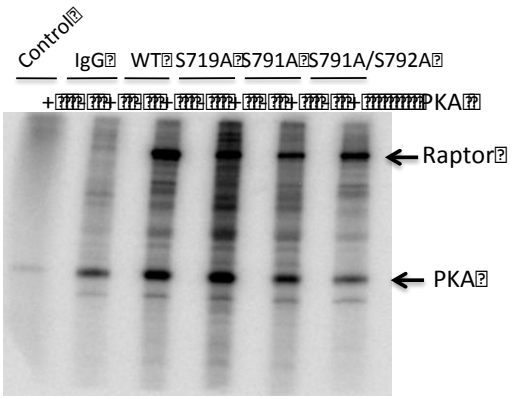

Fig. 3. PKA phosphorylates raptor in vitro. Control indicates on substrate was added in the in vitro kinase assay. IgG indicates immunoprecipitation of the control IgG while the rest is immunoprecipitation by raptor antibody. Arrows indicate the PKA autophosphorylation and raptor phosphorylation.

mTORC1 inhibition by osmotic stress

As a major cell growth regulator, mTORC1 has to integrates a wide range of signals, both stimulating and inhibitory, to regulate cell growth. Stress condition, such as osmotic stress, rapidly and potently inhibits mTORC1 (Fig.4A). mTORC1 inhibition is observed within 3 minutes of sorbitol addition (an osmotic stress). We hypothesize that protein kinases may mediate the osmotic signal to mTORC1 inhibition. In order to investigate the mechanism of osmotic stress induced mTORC1 inhibition, we screen the human kinome for kinases that when overexpressed can inhibit mTORC1. We found that expression of the NLK kinase potently inhibits mTORC1 (Fig.4B). This inhibition depends on the protein kinase activity of NLK (Fig.4B). To determine the in vivo function of NLK in osmotic response, we used the CRIPR gene editing technology to delete NLK in HEK293 cells [14]. We obtained two independent NLK knockout clones (Fig.4C). Interestingly, sorbitol treatment cannot inhibit mTORC1 in the NLK knockout cell lines (clones1-8 and 2-12). These results show that osmotic stress acts through NLK to regulate mTORC1. We will investigate the mechanism of NLK in cellular osmotic sensing and mTORC1 inhibition.

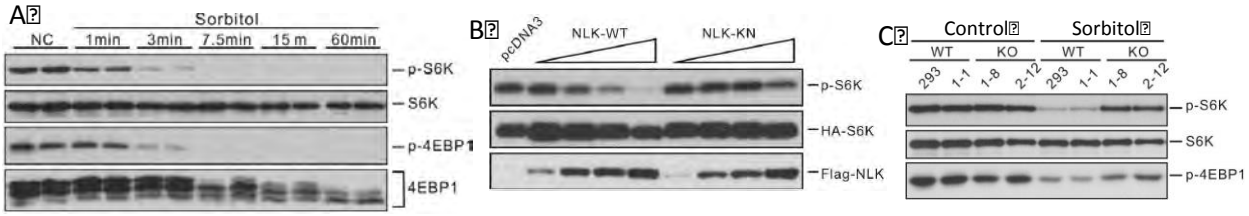

Fig.4. A. Osmotic stress (sorbitol treatment) rapidly inhibits mTORC1. **B.** NLK overexpression inhibits mTORC1 in a manner requiring kinase activity. NLK-WT and NLK-KN represent wild type and kinase inactive mutant, respectively. **C.** NLK plays a critical role in mTORC1 inhibition by osmotic stress. NLK wild type cells (WT, 293 and 1-1, which is a clone without NLK deletion) and NLK knockout cells (KO, 1-8 and 2-12) were treated with sorbitol for 7.5 minutes. mTORC1 activity was determined by western blotting for phosphorylation of S6K and 4EBP1.

Key Research Accomplishments

We have demonstrated that PKA mediates the effect of cAMP in mTORC1 inhibition. We also showed that cAMP suppresses the ability of both leucine and glutamine to stimulating mTORC1. Furthermore, PKA directly phosphorylates raptor. Finally, we showed that NLK mediates the osmotic effect on mTORC1 inhibition.

Conclusion

We have made significant progress on understanding the mechanism of mTORC1 regulation, particularly the mechanism of mTORC1 inhibition under unfavorable conditions. We showed that PKA mediates the inhibitory effect in response to G-protein coupled receptors that activate cAMP. A possible mechanism is proposed that PKA phosphorylates raptor to inhibit mTORC1. This phosphorylation may affect mTORC1 subcellular localization and/or directly reduce mTORC1 kinase activity. We also discovered the NLK protein kinase plays an important role in mediating the osmotic stress-induced mTORC1 inhibition. Finally, we have further advanced the molecular understanding of amino acids in mTORC1 activation by demonstrating that leucine and glutamine act through Rag GTPases and Arf1, respectively, to stimulate mTORC1.

Publications, Abstracts, and Presentations

1. Jewell, J.L., Kim, Y.C., Russell, R.C., Yu, F.X., Park, H.W., Plouffe, S.W., Tagliabracci, V.S., and Guan, K-L. (2015) Differential Regulation of mTORC1 by Leucine and Glutamine. Science 347, 194-198.
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Inventions, Patents and Licenses

Nothing to report

Reportable Outcomes

Nothing to report

Other Achievements

Nothing to report

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Appendices

Two publications.